

Optimization of intermittent hyperbaric oxygen exposures by duration of oxygen cycles

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Abstract

Objective: Intermittency is routinely used as a protective strategy against CNS and pulmonary hyperbaric oxygen (HBO₂) toxicity, however exact mechanisms of protection are not known. This study was designed to optimize intermittent HBO₂ exposures by variable duration of HBO₂ cycles.

Methods: Rats were exposed to O₂ at 2.8 ATA delivered either continuously for 6 h or intermittently with 10 min air breaks. Two intermittent paradigms were used; the first consisted of longer HBO₂ 60 min cycles at the beginning and shorter 30 min cycles at the end of exposure, while in the second paradigm, shorter cycles were followed by longer HBO₂ cycles. Effectiveness of intermittency was assessed by gene expression of inflammatory cytokines TNF- α , IL-1 β , IL-6 and heat shock protein 70 (HSP70) in the lungs at the end of exposures. The gene expression was compared with changes in respiration detected by continuous recording of respiration by a modified version of the whole-body plethysmography.

Results: All intermittent schedules inhibited lung inflammation compared with the continuous exposures. Efficacy of this inhibition was significantly better in intermittent groups starting with longer duration of HBO₂ cycles than in groups with reversed duration of HBO₂ cycles. Intermittent exposures starting with longer HBO₂ cycles also slowed down changes in respiratory frequency and tidal volume compared with the continuous exposure. HSP70 was increased in all exposure groups. It was significantly higher in continuous exposures and in intermittent group starting with longer duration of HBO₂ cycles than in the group starting with shorter HBO₂ cycles.

Conclusions: The higher benefit of the initially longer HBO₂ cycles could be in part explained by a higher oxidative stress and higher HSP70 expression in lungs compared with exposures with initially shorter HBO₂ cycles.

Key words:

Heat shock proteins;
Hyperbaric oxygen;
Intermittency;
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Introduction

Hyperbaric oxygen (HBO₂) is used in military and commercial undersea operations and recreational diving and it is an essential auxiliary treatment in the management of various acute and chronic pathological conditions. Currently there are 14 accepted indications by the Undersea and Hyperbaric Medical Society for the application of HBO₂ [1]. However, prolonged exposure to HBO₂ is also known to produce symptoms of pulmonary and central nervous system (CNS) O₂ toxicity [2, 3] that puts serious limits on its use. The pulmonary system is believed to be affected before the development of neurological symptoms at exposures to 100% O₂ at pressure 2.0 ATA or less while CNS HBO₂ toxicity is predominant at pressures higher than 2.8 ATA [2, 4]. The onset of HBO₂ toxicity and the extent of reversibility have

been shown to be dependent on various factors such as the absolute pressure of O₂, the duration of exposure, the partial pressures of O₂ and CO₂ and other factors [2, 4]. HBO₂ toxicity can be delayed or attenuated by systematic alternation of hyperbaric hyperoxic exposure intervals with brief intermittent intervals with lower PO₂ during exposures [2, 5-7].

This strategy of intermittency is routinely used for protection against CNS and pulmonary HBO₂ toxicity to enable longer total exposures to 100% O₂ at elevated pressures. Despite its routine clinical use, intermittency's exact mechanisms of protection and optimal exposure schedules are not known. Several curve-fitting models have been developed [8, 9] to define the optimal intermittent exposure paradigm based on experimental data in animals

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14. ABSTRACT <p>Objective: Intermittency is routinely used as a protective strategy against CNS and pulmonary hyperbaric oxygen (HBO2) toxicity, however exact mechanisms of protection are not known. This study was designed to optimize intermittent HBO2 exposures by variable duration of HBO2 cycles. Methods: Rats were exposed to O2 at 2.8 ATA delivered either continuously for 6 h or intermittently with 10 min air breaks. Two intermittent paradigms were used; the first consisted of longer HBO2 60 min cycles at the beginning and shorter 30 min cycles at the end of exposure, while in the second paradigm, shorter cycles were followed by longer HBO2 cycles. Effectiveness of intermittency was assessed by gene expression of inflammatory cytokines TNF-α, IL-1β, IL-6 and heat shock protein 70 (HSP70) in the lungs at the end of exposures. The gene expression was compared with changes in respiration detected by continuous recording of respiration by a modified version of the whole-body plethysmography. Results: All intermittent schedules inhibited lung inflammation compared with the continuous exposures. Efficacy of this inhibition was significantly better in intermittent groups starting with longer duration of HBO2 cycles than in groups with reversed duration of HBO2 cycles. Intermittent exposures starting with longer HBO2 cycles also slowed down changes in respiratory frequency and tidal volume compared with the continuous exposure. HSP70 was increased in all exposure groups. It was significantly higher in continuous exposures and in intermittent group starting with longer duration of HBO2 cycles than in the group starting with shorter HBO2 cycles. Conclusions: The higher benefit of the initially longer HBO2 cycles could be in part explained by a higher oxidative stress and higher HSP70 expression in lungs compared with exposures with initially shorter HBO2 cycles.</p>		
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and humans. These models provided a good fit for some intermittent schedules, however in many cases predictions made by models differed significantly from experimental data. In general, an autocatalytic model [8] and a Cox proportional hazard model [9] were similar for steep mortality curves, whereas the Cox model's predictions fit better than the autocatalytic model for more shallow curves. The most significant variations from predictions of the Cox model were underestimations of survival times that occurred at the highest pressure they studied (4 ATA)[9]. On the other hand, the autocatalytic model predicted that O₂ toxicity is greater for long shallow exposures than for short deep ones, which is not always empirically true [8]. Apparently, the mechanisms of HBO₂ toxicity and recovery are more complex than either model describes, or one equation cannot be applicable to all conditions, such as toxicity, steady state and recovery [10].

Triggering tolerance or toxicity may depend on the same factors, such as free radical generation, activation of cellular signaling, and accumulation of stress response-proteins, among other mechanisms. The cellular formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered to play a key role in the toxicity of O₂; however, they have also been suggested to mediate beneficial actions of O₂, such as stress response [11] activation of growth factors [12] or inhibition of inflammation [13]. Based on this concept, we surmised that an intermittency schedule in which the first (pre-air break) HBO₂ exposure was more rigorous, but was short of inducing toxicity, would provide a stronger stimulus to trigger the development of tolerance.

In our study we tested the hypothesis that the longer exposure and oxidative stress at the beginning of exposure will be more beneficial for the protective effect of intermittency against HBO₂ pulmonary toxicity manifested by inflammation, and alterations in lung mechanical function [6]. Therefore, rats were exposed to two different intermittent paradigms; the first consisted of longer HBO₂ cycles at the beginning of exposure and shorter cycles at the end, while in the second paradigm, shorter HBO₂ cycles in the beginning of exposure were followed by longer cycles later. The effectiveness of these intermittent schedules was assessed in lungs at the end of exposures by detection of: (1) gene expression of pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 as a marker of HBO₂ toxicity; and (2) by gene expression of heat shock protein 70 (HSP70) as a

potential protective factor against inflammation and against oxidative cell damage. Pulmonary respiratory function was determined via continuous recording of respiratory frequency and tidal volume during exposures using a modified version of whole-body plethysmography (WBP).

Materials and methods

Animals

Adult male Sprague-Dawley rats weighing 150-200 g (Taconic Farms, Germantown, NY, USA) were used in all the dives. Animals were supplied with standard rat chow and water *ad libitum* and a 12:12 hour light-dark cycle was maintained for at least one week before exposures. During HBO₂ exposure food and water were withdrawn from the animals and the experiments were conducted at approximately the same time on each day of the study. All experiments were designed in compliance with the guidelines that are specified in the 'Guide for the Care and Use of Laboratory Animals' (Institute of Laboratory Animals Resources, National Research Council, National Academy Press, revised 1999). The animal protocol was approved by the Institutional Animal Care and Use Committee at Naval Medical Research Center.

Animal grouping

The animals were divided into three exposure groups and one control group. HBO₂ exposures were chosen based on our previous study demonstrating prolongation of survival time in rats exposed to 30 or 60 min cycles of HBO₂ with intermittent air breaks resulting in up to 6 h of cumulative O₂ time (Chavko et al 2008). Our aim was to optimize these exposure schedules for potential use in clinical and military exposures and dives. For this reason, alternation of 30 and 60 min HBO₂ cycles with 10 min hyperbaric air breaks was used in two intermittent exposure paradigms. All exposures were performed at 2.8 ATA, *i.e.*, within the range of commonly used therapeutic pressure, 1.8-2.8 ATA [14].

In Group 1 (n = 8), animals were continuously exposed to HBO₂ for 6 h as a positive control group. Two animals in Group 1 died before the end of exposure and were excluded from the study. In Group 2 (n = 10), the first intermittent paradigm, three 60 min HBO₂ cycles were followed by six 30 min HBO₂ cycles interrupted with 10 min hyperbaric air breaks. In Group 3 (n = 10), the second intermittent paradigm, six 30 min HBO₂ cycles were followed by three 60 min HBO₂ cycles, with 10 min hyperbaric air breaks (Table 1). In

addition, a negative control group of animals (Group C, $n = 10$) was exposed to air (21% O_2) at atmospheric pressure (1 ATA) for 6 h. Immediately after exposure, animals were sacrificed, lungs removed and processed for RT-PCR assay.

Measurement of lung inflammation and HSP70 by real-time PCR

A portion of each lung (from the right lower lobe) was excised and stored in RNAlater (Ambion, Austin, TX, USA) at 20°C and homogenized by a vortex. Total RNA was isolated from 5 lungs at each group by phenol extraction. First strand cDNA was prepared from 1 µg of total RNA by using iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) and oligo(dT) primer in a 20 µl reaction mixture. For real-time PCR, 50 ng first-strand cDNA was used in a total volume of 25 µl of the iQ SYBR Green Super Mix (BioRad) containing 200 nmol of each mRNA-specific primer for TNF- α , IL-1 β , IL-6 or HSP70. PCR reactions consisting of 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min were performed on an ABI Prism 7700 Sequence Detection System and quantified using the comparative threshold cycle method with β -actin as a housekeeping gene reference.

Measurement of pulmonary respiration

Due to their electrical components, commercially available WBP systems are incompatible with the potential fire/explosion hazards of exposure to several atmospheres of 100% O_2 . In addition, environmental factors, such as CO_2 buildup, temperature and humidity that can change during prolonged exposure in an enclosed space may influence HBO₂ toxicity.

We have, therefore, developed an intrinsically safe system for performing WBP in rats at 2.8 ATA of 100% O_2 (Fig.1). Our system is composed of a 465 ml polymerized methylacrylic, tubular chamber (2.6" ID x 3.1" OD) endcapped by cut acrylic rods. The chamber is secured to the endcaps with 3 aluminum hexagonal rods and bolts. A stainless steel expanded metal tray, framed by acrylic rings (3" OD) and Delron rods, serves the dual functions of false floor and grounding.

Table 1. Animal grouping and exposure schedules

Group	HBO ₂ exposure schedule at 2.8 ATA
C	Sham exposures (normobaric, normoxic)
1	Continuous exposure for 6 hours
2	60-60-60-30-30-30-30-30-30
3	30-30-30-30-30-30-60-60-60

The duration of HBO₂ cycles in intermittent Groups 2-3 is indicated in min. Cycles of HBO₂ exposures were interrupted by 10 min intermittent air breaks

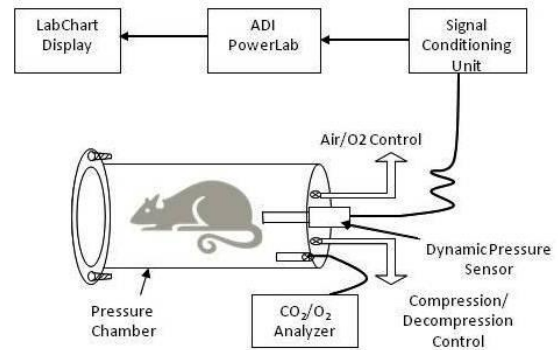


Figure 1. Schematic illustration of modified WBP system used for continuing recording of respiration during exposures to HBO₂.

There are 4 penetrations through one end plate: (1) a grounding penetrator, Conax Technologies, that touches the chamber grounding; (2) a port for a gas supply from cylinders routed through a Tescom regulator, Heise depth gauge, Swagelock pressure relief valve and N₂ purge capability; (3) a chamber exhaust port with a flowmeter and a sensor for O₂/CO₂ measurement (Series 9500, Alpha Omega Instruments Corp., Cumberland, RI, USA); and (4) a pressure sensor port plugged with a high intensity microphone (Model 106B, PCB Piezotronics Inc., Depew, NY, USA). The output of the microphone is conditioned by a signal processing module (ICP Sensor Signal Conditioner, Model 480E09, PCB Piezotronics Inc.) which is channeled into an eight channel PowerLab (Model ML8730, ADInstruments, Colorado Springs, CO, USA) data acquisition system and it is displayed on a LabChart window (Fig.1).

At the start of each experiment an awake, unrestrained rat was placed inside the chamber and the chamber was flushed with a continuous flow of air. The system enables conducting of experiments on four separate chambers in parallel. To minimize effects of fluctuations in the conditions of exposure, the placement of animals into the individual chambers was randomized irrespective of the experimental group. Fig.2 shows a snippet of pressure recording inside the chamber as a function of the rat's respiration. The inhaled volume of air which undergoes conditioning in the respiratory passages causes an expansion of the chest wall. This expansion leads to a build-up of pressure inside the chamber indicated by an upward movement in the signal during inhalation. As the rat exhales, the chest wall relaxes leading to a reduction in the pressure inside the chamber and a downward movement is recorded in the signal

during exhalation. This sequence of events was confirmed by video recording of the chest wall movement during respiration. Exposures started with a 10 min pre-exposure to 100% O₂ at atmospheric pressure (1 ATA). This brief period of normobaric hyperoxia allowed the rats to acclimatize to the chamber, stabilized the pressure measurement system, and set-up a baseline for subsequent measurements. In the control group, the initial period of acclimatization was provided with normobaric air. The chambers were then compressed to 2.8 ATA at 1 atm per min with 100% O₂. The gas flow rate was set at 4.5 lpm which was adequate to (1) maintain chamber CO₂ levels of less than 0.18%, (2) enable complete switching between gases (O₂ and air) within 1 min, and (3) maintain ambient temperature and humidity inside the chamber. The concentration of O₂ was maintained at 99-100% during HBO₂ cycles and at 21% during intermittent air breaks.

Data processing and analysis

Respiratory activity was recorded continuously for the entire duration of exposures. Plethysmographic output was analyzed during the first five minutes and last five minutes of each 30 min HBO₂ exposure period, and in the case of 60 min exposure periods also for five minutes in the middle of the exposure. Frequency of respiration (*f*), and the respiratory pressure change (*P_T*) were calculated after averaging data from the five 1 min intervals of the 5 min segments for fifteen respiratory cycles. Tidal volume *V_T* was then calculated using the equation of Drorbaug and Fenn [15]:

$$V_T = \frac{P_T \times (V_k/P_k) \times (T_R(P_B - P_C))}{(T_R(P_B - P_C) - T_C(P_B - P_R))}$$

P_T – the pressure deflection associated with each tidal breath in millivolts (mV)

V_k – the volume of air injected into the animal chamber for calibration (ml)

P_k – the pressure deflection associated with injection of calibrating volume *V_k* (mV)

T_R – rat lung temperature in degrees K (310°K)

T_C – temperature of the chamber in degrees K (293°K)

P_B – barometric pressure (760 mmHg)

P_C – vapor pressure of water in chamber (10 mmHg); this was derived from *T_C*, assuming 95% saturation of the chamber gas with water vapor

P_R – vapor pressure of water in lung at 100% saturation (47 mmHg)

V_k/P_k was determined by injecting a known calibration volume of air into the chamber at normobaric (1 ATA) and at hyperbaric (2.8 ATA) pressures. It was estimated that the transducer output in mV was a linear function of injected

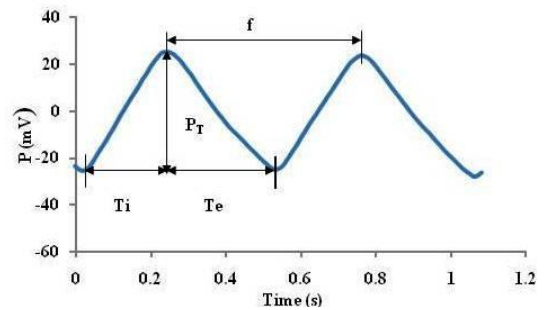


Figure 2; Pressure waveform as a function of respiration as recorded on LabChart. *f* – frequency of respiration; *P_T* – pressure change; *T_i* – inspiratory time; *T_e* – expiratory time.

volumes under both normobaric and hyperbaric conditions. For a given volume of injected air, the output signal was greater under the hyperbaric (2.8 ATA) condition than under the normobaric (1 ATA) condition, necessitating separate calibration parameters for each of the two pressure conditions. Regression analysis of the volume/transducer response curves for the normobaric (1 ATA) and hyperbaric conditions (2.8 ATA) used in this study gave calibration volumes of 0.0098 ml/mV at 1 ATA and 0.0028 ml/mV under pressure, respectively.

The hyperbaric chambers were continuously flushed with gas (air or oxygen) at a rate adequate to maintain the temperature and humidity inside the chamber at the room conditions. The above equation for calculation of *V_T* has been widely utilized by many investigators [16-18] and has been shown to characterize respiratory function in closed respiratory systems. However, in our modified version of WBP, the system was open, with a continuous flow of gas. In order to apply the same calculation in such an open system, two sets of calibrations were made: one with the chamber open and the other with the chamber closed. The difference in calculated pressure changes between the two conditions (open vs closed chamber) was found to be < 10%. The resulting estimation of *V_T* values was found to be in the range of values published previously in literature [19-21].

Statistical analysis

Gene expression data are expressed as means ± SD and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to compare statistical differences between the groups. Respiratory data was analyzed by a one-way ANOVA followed by a post hoc Dunnett's multiple comparison test with the *P* value < 0.05 as significant.

Results

Effect of HBO₂ on inflammatory cytokine and HSP70 gene expression

The inflammatory response in the lungs was assessed by the expression of three cytokines (TNF- α , IL-1 β and IL-6 mRNAs) as measured by quantitative PCR (qPCR). Gene expression of all three cytokines was markedly increased after continuous exposure to HBO₂ (Fig.3A-C). The use of intermittency (both intermittent schedules) mitigated these inflammatory cytokine responses compared with the continuous exposures. The efficacy of this mitigation was significantly better in intermittent Group 2, which started with longer durations of HBO₂ cycles than in intermittent Group 3, with reversed duration of HBO₂ cycles.

Gene expression of HSP70 in lungs was significantly increased in all exposure groups (Fig.3D) compared to negative controls (Group C). There was no significant difference between expression of HSP70 in the continuous exposure

group and intermittent Group 2 (longer cycles first). However, expression of HSP70 was significantly lower in intermittent Group 3 (shorter cycles first) compared with continuous exposure Group 1 and intermittent Group 2 (longer exposures first).

Effect of HBO₂ exposure on frequency of respiration (f) and tidal volume (V_T).

For simplicity, the graphical data for respiratory parameters do not include recordings made during the intermittent hyperbaric air breaks. Data were plotted to compare respiratory patterns in the continuous exposure group (Group 1) with those in intermittent Group 2 (longer HBO₂ cycles preceding shorter cycles) and intermittent Group 3 (shorter HBO₂ cycles preceding longer cycles). There was no change in respiratory pattern during the 6 h observation in the control group (Group C, animals breathing normobaric air) and that group was not included in subsequent comparative analyses.

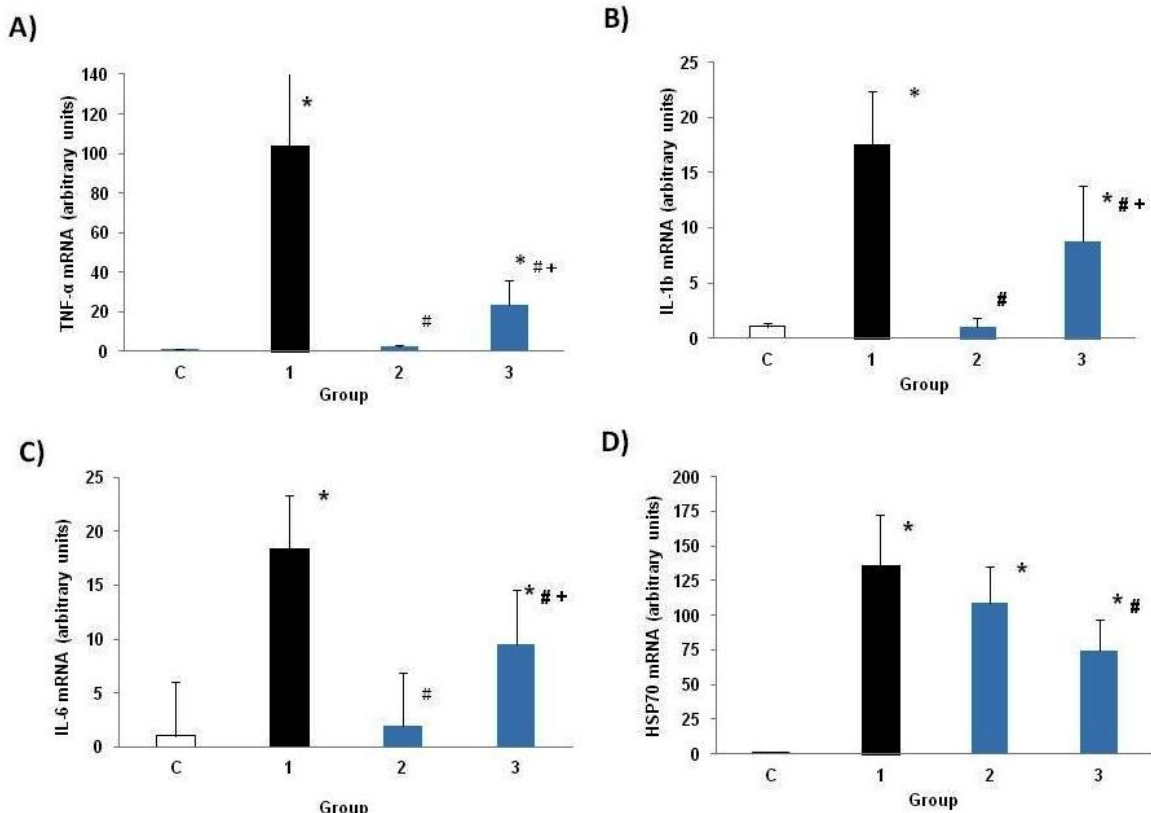


Figure 3. Inflammatory cytokine mRNAs expression in lungs of animals exposed to HBO₂. A) TNF- α ; B) IL-1 β ; C) IL-6 mRNA levels; D) HSP70 levels. Results are means \pm SD from 8-10 animals in each group.

*P < 0.05 compared with controls; #P < 0.05 compared with Group 1; +P < 0.05 compared with Group 3 (ANOVA followed by Tukey's test).

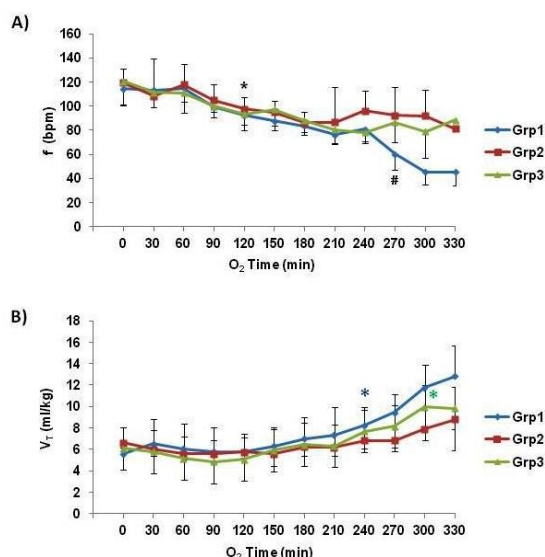


Figure 4. (A) Frequency of respiration (f) in rats exposed to 2.8 ATA O₂. The time axis shows the cumulative O₂ time without intermittent air breaks. Each point represents mean \pm SD from 8-10 animals. Frequency of respiration was significantly slower in all groups compared with the baseline at 120 min of O₂ exposure time. Starting from 270 min of exposure, the decrease in respiratory frequency in continuous exposure Group 1 was more pronounced than in intermittent Groups 2 and 3.

* $P < 0.05$ first decline compared with the baseline at the start of exposure (ANOVA followed by Dunnett's test). # $P < 0.05$ compared with Group 2 and 3 (t-test).

(B) Tidal volume (V_T) in rats exposed to 2.8 ATA O₂. The time axis shows the cumulative O₂ time without intermittent air breaks. Each point represents mean \pm SD from 8-10 animals. Increase in tidal volume was delayed in intermittent Group 2 (no significant increase) and Group 3 (300 min of O₂ time) compared with continuous exposure Group 1 (240 min of O₂ time).

* $P < 0.05$ first increase compared with the baseline level at the start of exposure (ANOVA followed by Dunnett's test).

A comparison of respiratory frequency among continuous and intermittent exposure groups over the entire duration of exposure is shown in Fig.4. There was a clear tendency for a decrease of respiratory frequency from the start to the end of HBO₂ exposure in all groups. Analysis of respiratory rate demonstrated a first significant decrease (compared to the baseline) in frequency of respiration between 150 and 180 min in all exposure groups (Fig.4). With continuation of exposure the frequency of respiration decreased significantly in Group 1 (continuous exposure) compared with Groups 2 and 3 (intermittent exposures).

The overall pattern of the changes in tidal volume during the entire duration of HBO₂ exposure is shown in Fig.4. It appears that for the first few hours of exposure there was a slight decrease or no

change in V_T in all exposure groups. After further exposure, V_T gradually increased with the slope of the increase depending on the exposure profile. A significant increase in V_T compared with the baseline was evident after 240 min of exposure in the continuous exposure group (Group 1). In Groups 2 and 3 the time for V_T increase was delayed (300 and 330 min respectively) in comparison with the Group 1.

Discussion

Exposure to high concentrations of oxygen has been demonstrated to cause alterations throughout the respiratory tract in humans and animals. The earliest morphologic changes seen in the lung in response to hyperoxic stress probably involve subtle changes in endothelial cell ultrastructure, which result in pericapillary accumulation of fluid. This stage of lung cell injury is associated with, or is rapidly followed by, accumulation of inflammatory blood cell elements in the lung and the release of soluble mediators of inflammation [22]. Several studies have demonstrated a correlation of the number of neutrophils obtained by lung lavage and the magnitude of injury [22, 23]. Short intermittent air breaks during HBO₂ exposures are known to extend pulmonary tolerance to HBO₂ as evidenced by extension of survival times in rodents [8, 9] and slower development of decrements in lung function in human [5, 6].

Analysis of experimental data with risk models linking probability of death to the accumulation of a putative toxic substance suggested that development of toxicity corresponded to autocatalytic reactions rather than to linear processes [8]. In general, the autocatalytic model predicts, and animal data show, that the risk of O₂ toxicity increases as the ratio of oxygen to normoxic time increases (*i.e.*, a 30-5 schedule is riskier than a 20-5 schedule) and that for any given on:off ratio, the risk increases as absolute cycle length increases (*e.g.*, for a stable 4:1 ratio, a 50 minute cycle length [40-10] is worse than a 12.5 minute cycle length [10-2.5]). While there is a general perception that longer air breaks provide improved benefit, the role of HBO₂ duration preceding intermittent breaks is not so obvious. Clark *et al* [24] measured visual and pulmonary indices of oxygen poisoning in two groups of subjects that were exposed intermittently on either a 60-15 or a 30-30 (min) pattern. The latter pattern was expected to provide greater extension of oxygen tolerance because it simultaneously halved the oxygen exposure period and doubled the

normoxic recovery interval. Unexpectedly, the toxic effects associated with the 30-30 pattern exceeded those for the 60-15 pattern. This is consistent with the results of this present study. It is also in agreement with the assumptions that a minimal O₂ time may be required for the benefits of intermittency to be realized [25] and that putative protective mechanisms are activated more effectively with higher pressures/durations of HBO₂ cycles [9].

It is well widely accepted that ROS mediate HBO₂ pulmonary and central nervous system toxicity manifested by changes in respiration and grand mal seizures. ROS and RNS participate in metabolic and transduction pathways as signaling molecules and depending on their concentration and cellular localization they may mediate both toxic and beneficial effects by modification of macromolecules and activation of different transduction or signaling pathways [26]. In the present study, we used gene expression of the inflammatory cytokines TNF- α , IL-1 β and IL-6 to characterize the development of HBO₂ toxicity in lungs of animals exposed to continuous exposure to HBO₂ and to different intermittent exposure schedules. A significant, many-fold increase in mRNA expression for all three cytokines was found to occur after continuous exposure to HBO₂. All intermittent schedules inhibited lung inflammatory activation. The major finding of this study was that intermittent exposures starting with longer duration of HBO₂ cycles were more efficient in mitigation of inflammation than the intermittent schedules starting with shorter HBO₂ cycles. The most significant differences were found in the TNF- α mRNA expression between exposure groups. TNF- α plays a critical role in initiating inflammation and enhancing the production of inflammatory mediators such as IL-6 which are thought to further promote lung injury [27]. The role of intermittency against HBO₂ toxicity is presumed to be associated with the activation of inducible forms of antioxidant enzymes such as manganese superoxide dismutase or heme oxygenase-1 [28, 29]. However, antioxidant enzymes are expressed in low levels or not rapidly enough to protect against injury.

Tolerance formation likely requires activation of other factors and mechanisms, because exposure to 50%, or 65% O₂ induced superoxide dismutase (SOD) activation but not tolerance [30]. It may include activation of stress proteins or activation of molecular pathways associated with cell tolerance to oxidative stress. Early induction of heat shock

proteins in response to hyperoxia is a well-known adaptive and protective mechanism against oxidative stress [31]. HSP70 is the most highly-induced stress protein in whole lungs and specific lung cells in response to a variety of stressors including hyperoxia, and it is central to the cytoprotective properties of the stress response [32]. The mechanism of protection against HBO₂ toxicity by heat acclimation is directly related to HSP72 level in brain [33]. Pretreatment with repetitive exposures to HBO₂ was shown to protect against ischemic neuronal damage by activation of HSP72 [34]. Our results demonstrate a robust activation of HSP70 expression in lungs following exposure to HBO₂. The extent of HSP70 activation was significantly higher in intermittent Group 2 (longer cycles first) than in intermittent Group 3 (shorter cycles first) corresponding well with the mitigation of inflammation. However, the level of HSP70 was about the same in both the continuous exposure group (Group 1) and in intermittent Group 2 (longer cycles first), indicating that the same degree of HSP70 overexpression can be associated with either tolerance or its absence. There are several possible interpretations of these findings with regard to the possible role of HSP70 in the development of tolerance to HBO₂ exposure:

- (1) HSP overexpression plays a role in the development of tolerance, but the tolerance mechanism is ultimately overwhelmed by the toxic effects of HBO₂ when the exposure is continuous and prolonged.
- (2) HSP overexpression plays a role in the development of tolerance, but other factors, unknown and unmeasured in these experiments are also critical.
- (3) HSP overexpression induced by HBO₂ exposure is epiphenomenal to the development of tolerance.

It appears that HSP70 expression is induced continuously during exposure to HBO₂ and could be related to the level of oxidative stress in the cells. According to interpretation (1) above, this HSP70 overexpression may up to some point protect against HBO₂ toxicity by inhibition of inflammation or by its molecular chaperone effect. With continuing exposure, the protective effect of HSP70 may be counterwhelmed by an excessive accumulation of ROS resulting in oxidation of macromolecules and development of toxicity. Intermittent breaks during HBO₂ exposure may protect against toxic levels of ROS accumulation and thus may maintain balance in favor of recovery from toxicity and development of tolerance. The

role of ROS in the development of HBO₂ pulmonary toxicity was demonstrated by the progressive increase in lipid peroxidation products in exposures to 3 ATA O₂ [35]. The higher level of HSP70 in Group 2 (longer cycles first) compared with Group 3 suggests that a threshold level of ROS at the beginning of exposure is required for an efficient stress response activation. This is in agreement with the recent observation that pretreatment with antioxidants abolished the protective effect of hyperoxia on zymosan induced inflammation in mice [13].

In accordance with interpretation (2) above, the lower overexpression of HSP70 in Group 3, which did develop tolerance, compared to the continuously exposed group (Group 1), which did not develop tolerance, suggests that the induction of HSP70 expression is not the only mechanism responsible for protection against HBO₂-induced toxicity by intermittency.

The effect of continuous exposure to HBO₂ on pulmonary function over a range of O₂ pressures was summarized by Clark and Thom [6]. In general, the pattern of pulmonary function deficit varies in different combinations of oxygen pressure and exposure time. It also appears that lung mechanical function is impaired earlier and more significantly than pulmonary gas exchange. To compare activation of inflammation/HSP with pulmonary function, respiratory parameters *f* and *V_T* were continuously recorded by WBP. The WBP has long been used to quantify ventilatory parameters in a variety of small animals under normobaric conditions [21, 36, 37]. The classical plethysmograph chamber is a closed system with no gas flow through the chamber during recording of respiration for duration of 5 to 10 s. In order to avoid the electrical hazards of commercially available closed systems, and to optimize our environmental control (CO₂, temperature, humidity) during prolonged exposures we designed and built the unique open WBP system described in the Methods section. Despite some small differences in calibration in open versus closed system, the normobaric ventilatory parameters in our open system were comparable with those published before in closed WBP [19].

The respiratory deficiency during HBO₂ includes decreases in vital capacity, respiratory frequency, pulmonary compliance, and respiratory responsiveness to hypercapnia [2]. The breathing pattern in both normobaric and hyperbaric hyperoxia is affected in a similar way; decreased breathing frequency and increased tidal volume

[20, 38]. In all exposures we observed decrease in *f* and delayed increase in *V_T*. The important finding in the study was a correlation between extent of pulmonary inflammation and changes in respiratory pattern. Basically, the intermittency schedule starting with longer HBO₂ cycles delayed symptoms of respiratory deficiency compared with continuous exposure. This was demonstrated by slower decline in respiratory frequency and by delayed increase in tidal volume. On the other hand, differences between the two intermittent schedules were not as dramatic as observed in suppression of lung inflammatory response. There are several interpretations of the O₂ effect on breathing such as desensitization of the carotid chemoreceptors [39] or C-fiber stimulation [40]. Denervation of the lung [41] and sympathectomy and deafferentation of the carotid bifurcation [42] caused similar changes as observed after hyperoxia, *i.e.* increase in *V_T* and a decrease in *f*. It was suggested that a similar mechanism may contribute to the effect of hyperoxia on breathing pattern [20]. The mechanism for this may involve the carotid body desensitization which could be related to the accumulation of ROS during exposure and attenuation of O₂ chemosensing mechanisms in the carotid body.

In summary, results of this study show that: (1) lung inflammation in prolonged exposure to HBO₂ can be significantly inhibited by intermittent air breaks of 10 min duration; (2) longer HBO₂ cycles at the beginning of exposures and shorter cycles at the end provides more efficient anti-inflammatory effect than vice-versa; (3) activation of inflammatory lung response correlates with changes in respiratory pattern (frequency and tidal volume) during exposure to HBO₂, and (4) higher HSP70 induction may be in part responsible for more beneficial effect of longer HBO₂ cycles compared with shorter cycles at the beginning of exposures.

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Competing interests

The authors declare that they have no conflicts of interest.

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